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(71) *Sökande* *Pharmacia & Upjohn AB, Uppsala SE*
Applicant (s)

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For the Patent- and Registration Office

Åsa Dahlberg

Åsa Dahlberg

Avgift
Fee

PATENT- OCH REGISTRERINGSVERKET
SWEDEN

Postadress/Adress
Box 5055
S-102 42 STOCKHOLM

Telefon/Phone
+46 8 782 25 00
Vx 08-782 25 00

Telex
17978
PATOREG S

Telefax
+46 8 666 02 86
08-666 02 86

Methods for diagnosis

Technical field

The present invention relates to methods for the diagnosis of allergic bronchopulmonary aspergillosis (ABPA) and recombinant allergens to be used in the methods.

Technical Background

Allergic bronchopulmonary aspergillosis (ABPA). Allergic bronchopulmonary aspergillosis is the most severe allergic complication caused by Aspergillus species, mainly A. fumigatus. ABPA is the result of hypersensitivity to Aspergillus-antigens mainly in patients suffering from long-standing atopic asthma (8-12) or cystic fibrosis (16-19). Although originally considered as a rare disease (13), ABPA is currently recognized with much greater frequency. ABPA with varied clinical presentations has been reported to occur in about 15% of the asthmatic patients sensitized to A. fumigatus (14,15), while in patients with cystic fibrosis the reported incidence varies from 10 to 35% (16,17). ABPA has been described as an immune disease that ranges from asthma to fatal destructive lung disease with defined clinical, serological, radiological and pathological features (8,18-22). Because of its severity ABPA should be ruled out in patients with chronic asthma or cystic fibrosis exhibiting immediate cutaneous reactivity to A. fumigatus (8). The diagnostic criteria for ABPA are asthma or cystic fibrosis, history of roentgenographic infiltrates (in most cases), immediate cutaneous reactivity to A. fumigatus extracts, elevated total serum IgE, precipitating antibodies to A. fumigatus, peripheral blood eosinophilia, elevated specific serum IgE and IgG to A. fumigatus as compared to sera from patients with asthma and cutaneous reactivity to Aspergillus, but without ABPA, and proximal (central) bronchiectasis with normal tapering of distal bronchi (23-25). In cases where all criteria are present, diagnosis is readily made (26). However, all of the eight criteria are rarely present at the same time even in classic ABPA-patients with central bronchiectasis. With exception of bronchiectasis and to some

extent elevated specific serum IgE and IgG to A. fumigatus, none of the diagnostic criteria are specific for ABPA (26). Furthermore, pulmonary infiltrates and central bronchiectasis are commonly detected in patients suffering from cystic fibrosis also in the absence of sensitization to A. fumigatus, which makes a diagnosis of ABPA in patients with cystic fibrosis even more difficult (16). Therefore, serologic identification of ABPA has a greater diagnostic potential, but is, however, hampered by the lack of standardized, reliable fungal extracts (5,7,27-29).

Aspergillus fumigatus antigens. The major problem in the immunodiagnosis of diseases related to A. fumigatus stems from the antigenic compexity of the fungus. Antigen/allergen extracts of A. fumigatus contain hundreds of different proteins (6,30,31), of which a limited subset are able to bind human serum IgE (6,32,33,35). The fungus has been reported to produce more than 40 IgE-binding components which generate complex IgE-binding patterns when extracts are examined by Western blot analysis using sera from allergic individuals (32,33). To make the picture even more complicated, serum IgE from different patients recognize highly variable patterns of fungal proteins (6,36). In the case of patients suffering from ABPA, depending on the stage of the disease, different allergenic "fingerprints" may be obtained with serum of the same patient taken at different times, even if fungal extract from the same batch is used (36,37).

It has been suggested to use purified native allergenic components instead of crude allergen extracts for diagnosing ABPA (79). Recombinant A. fumigatus allergens with connections to ABPA have been described earlier (71,83).

The inventors are named authors in a number of articles about recombinant allergens from A. fumigatus (cloning and expression: 39,43,49,51,52,82, and diagnostic use: 59,66,32,71,76,81).

The objectives of the invention.

The main objective of the invention is to provide improved methods for diagnosis of ABPA.

One subobjective is to provide in vitro diagnostic methods that have the sufficient specificity and sensitivity for diagnosis of ABPA.

A second subobjective is to provide well-defined allergen preparations that can be used for the diagnosis of ABPA both in vitro and in vivo, including immuncassay and skin reactivity measurement methods, respectively.

The invention

The first major aspect of the invention is a method for diagnosis of allergic bronchopulmonary aspergillosis (ABPA). This aspect is characterised in using as a reagent an ABPA-related recombinant allergen, i.e. a recombinant allergen carrying an epitope against which antibodies of various Ig classes/ sub-classes, such as of the IgE class or total IgG or IgG subclasses (IgG1, IgG2, IgG3 and IgG4) can be detected so that an ABPA condition in a patient can be differentiated from allergic sensitization to A. fumigatus, which is particularly useful in patients suffering from cystic fibrosis.

The concept of ABPA-related recombinant allergens includes any recombinant allergen, irrespective of origin, having the above-mentioned antibody binding feature permitting the differential diagnosis indicated. It encompasses in particular ABPA-related recombinant allergens derived from A. fumigatus and their ABPA-related fragments. For ABPA-related recombinant allergens cloned from A. fumigatus, the concept encompasses ABPA-related allergens and fragments derived from other sources, having one or more ABPA epitopes in common with an ABPA-related allergen from A. fumigatus. At the priority, date rAsp f2 and rAsp f4 and their fragments, as defined above, were considered to be the most useful ABPA-related allergens. Various derivatized forms retaining the ability to bind antibodies, as defined for ABPA-related recombinant allergens, are also included.

Various subaspects include in vitro and in vivo testing protocols as described below.

The second major aspect of the invention is novel ABPA-related recombinant allergens binding to human IgE present in ABPA patients and useful in the first aspect of the invention.

Various subaspects of this second major aspect of the invention are apparent from the below and encompasses derivatized forms including but not limited to underivatized, insolubilized and labelled ABPA-related allergens.

Another aspect of the invention is the use of ABPA-related allergens for hyposensitization treatment as done for other allergens.

Cloning of allergens from Aspergillus fumigatus.

The cloning strategy utilized phagemid pComb3 (47) and the ability of the leucine zipper proteins Jun and Fos to associate with each other (74,48,74,75).

A modified gIII product, obtained by fusing the DNA encoding the jun leucine zipper flanked by cysteine residues, N-terminal to the viral coat protein was expressed from a LacZ promotor and secreted into the periplasmic space of E. coli by a pelB leader peptide, thereby being structurally incorporated into phage particles during infection with helper phage (49). Using a second LacZ promotor of the phagemid, the fos leucine zipper domain, flanked by cysteine residues, co-expressed as N-terminal fusion peptide to cDNA protein products of A. fumigatus, was secreted into the periplasmic space of E. coli using the pelB leader peptide (50). Through Jun-Fos heterodimerization and disulfide bond formation, the gIII-Jun fusion protein incorporated into phage particles provides a covalent link to phage surface for random recombinant cDNA products with the Fos leucine zipper attached N-terminally (48,49). The phagemid pJuFo which contains the described elements (49,51,52), allows expression and display of cDNA libraries, in this case encoding shot-gun cloned A. fumigatus peptides/proteins, on phage surface and application of the powerful screening technology based on biopanning procedures used for other filamentous phage systems (46,47). The key of success in cDNA cloning from libraries displayed on phage surface

lies in the screening strategy used. The most important factor to be considered is that the ligand used to select phage should be tagged or immobilized in a way allowing the ligand to retain its native conformation (46). It must be taken into account that proteins, when directly immobilized to a solid phase by hydrophobic interaction, may lose biological activity due to alterations in the three-dimensional structure (54,55). In general the known or expected characteristics of the ligand will dictate the procedure used for ligand immobilization. For the isolation of allergens recognized by serum antibodies, the use of capture antibodies has proven to be very effective for different reasons. First, monoclonal antibodies raized against the immunoglobulin ε constant domains Cε2, Cε3 or Cε4 do not interfere with the antigen binding site of the antibody. Second, a surface coated with such anti-IgE antibodies will be able to immobilize selectively IgE antibodies from serum of allergic patients. Therefore, after washing away interacting and cross-reacting serum antibodies of other isotypes together with all other serum components, a specific surface able to adsorb only phage displaying IgE binding molecules will be obtained (51-53).

The application of pJuFo to display cDNA products and select phages from a library constructed using mRNA from A. fumigatus (39,51,52) yielded a wide variety of phage clones able to bind IgE antibodies from sera of patients sensitized to A. fumigatus (table 1).

Table 1. Typical enrichment of phage from a cDNA phage display library by biopanning. Phage displaying cDNA-products from an A. fumigatus expression library were applied to a single well of a microtitre plate coated with human serum IgE (51,52). After adsorption and extensive washing adherent phages were eluted and used to infect E. coli for a further round of phage growth and selection.

Round of panning	Phage input[a]	Phage output[b]	Enrichment factor[c]	IgE-specific phage phage tested[d]
1	1.8×10^{11}	4.5×10^{3}	5.6×10^{-6}	0/10

2	$8.6x10^{10}$	$2.4x10^{4}$	$5.6x10^{-5}$	0/10
3	$9.3x10^{10}$	$4.6x10^{4}$	$3.8x10^{-5}$	0/10
4	$6.5x10^{10}$	$1.1x10^{5}$	$1.9x10^{-4}$	1/10
5	$1.8x10^{11}$	$3.8x10^{6}$	$2.3x10^{-3}$	8/10
6	$2.1x10^{11}$	$8.4x10^{6}$	$4.0x10^{-3}$	10/10
7	$9.4x10^{10}$	$5.8x10^{7}$	$5.6x10^{-2}$	10/10

a) Number of phage applied to a single well of a microtitre plate.

b) number of phage eluted from the well after washing.

c) Percentage yield of phage from each round of panning (%yield = [No. of phage eluted x 100]/[No. of phage applied]).

d) Single colonies from plates used to titrate phagemids were grown in liquid culture, phage induced and purified. Phage coated directly to microtitre plates were tested for IgE binding capacity by an IgE-specific ELISA (66). IgE-specific phage represents phage able to bind serum IgE/number of phage tested.

Compared with screening of λ-libraries immobilized on solid phase supports, the screening procedure for cDNA libraries displayed on the surface of filamentous phage has several advantages. Capturing serum IgE with an immobilized anti-IgE antibody generates a homogenous surface with immobilized IgE which does not become denatured (56,57) and therefore retain the full antigen binding capacity. The most important advantage results from the fact, that the phage library is kept in a liquid phase, where only phage with affinity to the ligand are retained on the solid phase after washing (47,53). Desorbed phage can be used to infect E. coli in order to amplify phage with affinity for the ligand. Therefore, successive rounds of phage growth and selection allow enrichment of phage displaying proteins with affinity for the ligand (table 3). After selection of candidate phage clones displaying proteins with IgE binding properties, phage particles produced from 10 ml culture can be precipitated (47) and samples of 1010-1013 phage particles of each candidate clone analysed by SDS-PAGE under reducing conditions, followed by transfer to nitrocellulose membranes (49,51). After blocking in order to saturate free binding sites on the nitro-cellulose

sheet, membranes are incubated with patient serum diluted 1:10 as "first antibody" and mouse anti-human IgE mAb as second antibody can be used to visualize binding of IgE to the cDNA product originally present on the phage surface. Western blots can easily be developed using non-radioactive systems and horse-radish-peroxidase-conjugated goat-anti mouse Ig as detection system. The apparent molecular mass of the IgE-binding proteins enriched from an A. fumigatus cDNA library displayed on phage surface was in the range of 10 to more than 50 kDa. Nucleotide sequence determination (58) of some cDNA-inserts differing in size and restriction pattern revealed that they encode different proteins as deduced from the open reading frames.

Table 2. Main characteristics of phages isolated from an A. fumigatus cDNA library displayed on a phage surface and subjected to selective enrichment using patient serum IgE as ligand.

Phage No	Insert[a] length bp	Mw of the[b] protein(kDa)	Expression[c] mg/l culture	IgE-binding[d] frequency	Preliminary[e] designation	Skin Test[f] reactivity
2	1103	40	36	<50%	rAsp f4	+
7	854	10	65	<50%	rAsp f7	nt
19	751	28	220	<50%	rAsp f2	+
28	616	21	38	<50%	rAsp f3	+
38	1123	33	25	<50%	rAsp f9	
nt						
46	686	18	140	<50%	rAsp f1/a[g]	+
48	1270	42	45	<50%	rAsp f5	+
51	978	34	29	<50%	rAsp f10	nt

a) Determined from the nucleotide sequences

b) Estimated from polyacrylamide gels

c) Produced as inclusion body protein after subcloning of the fragments into pDS76/RBSII, 6xHis (62). The yields represent mg of Ni^{2+}-chelate affinity purified protein per litre culture (60,61).

d) Sera from 54 patients with ABPA and from 35 individuals sensitized to A. fumigatus were tested for the presence of specific IgE to the recombinant proteins. IgE-binding frequency was assigned to major (>50%) or minor (<50%) allergens (41).

e) Nomenclature not officialized.

f) Skin test reactivity determined as described (66), nt = not tested.

g) This allergen has been previously described.

Production of recombinant allergens in E. coli.

Illustrative examples of production methods will now be given for two ABPA-related recombinant allergens cloned from A. fumigatus, tentatively designated rAsp f2 and rAsp f4.

rAsp f2: DNA encompassing the coding sequence of rAsp f2 was cloned into an expression vector under the transcriptional control of the T7 promoter (78). The construct was designed in such a way that one methionine residue was added at N-terminal end of the allergen amino acid sequence, while at the C-terminus the eight-residue stretch -VEHHHHHH was added, of which the six consecutive histidine residues serve as an affinity tag for metal-chelate affinity chromatography (61). After sequence confirmation, the construct was transferred to E. coli BL21[pT7POL23] (77), in which synthesis of the T7 RNA polymerase can be induced by raising the temperature of the growing culture to above 37°C. To produce rAsp f2, 1 liter of LB medium containing an appropriate complement of antibiotics was inoculated with 1 ml of an overnight starter culture grown at 30°C. After approximately 3 hrs of growth at 30°C, at an $OD_{...}$ of 0.7, the temperature of the culture was shifted to 42°C in order to induce expression. After 4 hrs of of incubation at inducing temperature, cells were harvested by centrifugation and resuspended in 50 ml of ice-cold 20 mM Tris-HCl pH 8.0 containing 0.5 M NaCl and 5 mM imidazol (resuspension buffer). The cells were disrupted by sonication and insoluble debris removed by centrifugation. The supernatant, containing the overexpressed allergen, was passed through a 0.22 µm filter to remove remaining particulate material and loaded onto an assembly of two serially

connected 5 ml HiTrap Chelating columns (Pharmacia Biotech AB, Uppsala, Sweden) previously charged with Ni^{2+} and equilibrated with resuspension buffer. The colum assembly was washed first with 50 ml of resuspension buffer, then with 50 ml of resuspension buffer supplemented with Imidazol to 60 mM. To elute rAsp f2, a 30 ml linear gradient of 60-500 mM imidazol in 20 mM Tris-HCl pH8.0/0.5 M NaCl was applied while 1 ml fractions were collected and analysed by SDS-PAGE. Fractions containing rAsp f2 were pooled and subjected to gel filtration through a Superdex 200 column (Pharmacia Biotech AB, Uppsala, Sweden) equilibrated and eluted with 0.15 M NaCl. Fractions containing rAsp f2 were pooled and concentrated using an Amicon cell fitted with a YM5 membrane. Final yield of purified rAsp f2 from one liter of bacterial culture was 23 mg.

rAsp f4: DNA encompassing the coding sequence of rAsp f4 was cloned into an expression vector under the transcriptional control of the T7 promoter (78). The construct was designed in such a way that the 11-residue stretch MRGSHHHHHHM- was added to N-terminal end of the allergen amino acid sequence, of which the six consecutive histidine residues serve as an affinity tag for metal-chelate affinity chromatography (61). No amino acid addition was made at the C-terminal end of the protein. After sequence confirmation, construct was transferred to E. coli BL21[pT7POL23] (77), in which synthesis of the T7 RNA polymerase can be induced by raising the temperature of the growing culture to above 37°C. To produce rAsp f4, 1 liter of LB medium containing an appropriate complement of antibiotics was inoculated with 1 ml of an overnight starter culture grown at 30°C. After approximately 3 hrs of growth at 30°C, at an $OD_{...}$ of 0.7, the temperature of the culture was shifted to 42°C in order to induce expression. After 4 hrs of of incubation at inducing

temperature, cells were harvested by centrifugation and resuspended in 50 ml of ice-cold 20 mM Tris-HCl pH 8.0 containing 0.5 M NaCl. The cells were disrupted by sonication and insoluble material including rAsp f4 protein was collected by centrifugation. The insoluble material was washed twice by resuspension in 20 mM Tris-HCl pH 8.0 containing 2 M Urea, 0.5 M NaCl and 2% Triton X-100, followed by centrifugation. Partially purified rAsp f4-containing inclusion bodies were extracted for 45 minutes at room temperature in 70 ml of 20 mM Tris-HCl pH8.0 containing 6 M guanidinium hydrochloride, 0.5 M NaCl, 5 mM Imidazol and 1 mM 2-mercaptoethanol (extraction buffer). The extract was clarified by centrifugation and remaining particulate material removed by passage through a 0.22 µm filter. The clarified extract, containing the overexpressed allergen, was loaded onto an assembly of two serially connected 5 ml HiTrap Chelating columns (Pharmacia Biotech AB, Uppsala, Sweden) previously charged with Ni^{2+} and equilibrated with extraction buffer lacking 2-mercaptoethanol. The column assembly was washed first with 50 ml of extraction buffer, then with 50 ml of 6 M urea in 20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 20 mM Imidazol and 1 mM 2-mercaptoethanol (urea wash buffer). In order to renature the immobilized rAsp f4, a 960 ml linear gradient was applied, from urea wash buffer to 20 mM Tris-HCl pH8.0 containing 0.5 M NaCl, 20 mM Imidazol and 1 mM 2-mercaptoethanol (renaturation buffer). To elute rAsp f4, a 30 ml gradient of 20-1000 mM imidazol in renaturation buffer was applied while 1 ml fractions were collected and analysed by SDS-PAGE. Fractions containing rAsp f4 were pooled and subjected to gel filtration through a Superdex 75 column (Pharmacia Biotech AB, Uppsala, Sweden) equilibrated and eluted with 0.15 M NaCl. Fractions containing rAsp f4 were pooled and concentrated using an Amicon cell fitted with a YM10

membrane. Final yield of purified rAsp f4 from one liter of bacterial culture was 34 mg.

Production has also been carried out with the vector described by Hochli et al (60-63).

Analysis of cDNA inserts

Only inserts coding for peptides/proteins relevant for the diagnosis of ABPA will be discussed.

rAsp f2 (SEQ ID NO 1). A clone containing an insert of 751 base pairs with an open reading frame of 624 base pairs revealed a strong homology with nucleotide sequences encoding superoxide dismutases. The 3'-noncoding region had a polyadenylated tail of 24 base pairs. The deduced amino acid sequence of this cDNA clone (SEQ ID NO 1) was homologous to manganese SOD, showing the highest sequence identity of 48-52 % to the human, fruit fly, gum tree, yeast, E. coli, and Mycobacterium leprae enzymes. Apparently the A. fumigatus MnSOD displays a similar high degree of sequence identiy to MnSODs from a wide variety of phylogenetically distant organisms (43). Multiple sequence alignment shows that the A. fumigatus MnSOD (rAsp f2) shares high homology with human MnSOD (51.8% identity, 67.2% homology). IgE raised against A. fumigatus MnSOD is detected predominantly in sera of patients suffering from ABPA. Therefore MnSOD could be a candidate for a serologic differential discrimination between ABPA and A. fumigatus allergy (see below). Notably, both recombinant A. fumigatus and human MnSOD induce proliferation in peripheral blood mononuclear cells of A. fumigatus allergic subjects with detectable levels of specific IgE to A. fumigatus MnSOD. Moreover, both the fungal and human recombinant MnSODs elicited Type I skin reactions in individuals sensitized to the fungal enzyme, providing evidence for auto-reactivity to human MnSOD in allergic individuals sensitized to the environmental A. fumigatus allergen (43).

rAsp f4 (SEQ ID NO 2). This was the second recombinant ABPA-related allergen discovered in our screening system. The clone contained an isert of 1103 base pairs with an open reading frame

of 858 base pairs. Its deduced amino acid sequence does not share significant homology to any known protein. The gene product encoded by the used cDNA was only characterized by the function for which it was selected: IgE binding.

In vivo tests utilizing recombinant allergens.

These are mainly illustrated by skin prick tests in which a small amount of a solution of an allergen is inserted into the dermis of an individual whereupon a wheal reaction occurs around the place for administration.

One protocol for skin prick tests of the invention implied that a recombinant allergen was dissolved in 0.9% physiological saline as a diluent at an end concentration of 100 µg/ml. 20 µl of these solutions were placed on the patient's forearms. Thereafter the skin was pricked with a sterile needle, which was entered into the epidermis at a degree angle and lifted up to elevate a small portion of the epidermis (38). The needle was discarded after the application of each solution to avoid contamination. The test sites were placed 3 to 4 cm apart to avoid false positive results.

For intradermal tests, an allergen solution (100 µg/ml) were diluted at serial 10-fold dilutions and applied at concentrations starting from 10-4 µg/ml to 10 µg/ml. For testing the solutions (100 µl) were injected on the patients' backs starting from the solution with lowest concentration resulting in a size of the wheal of half the size of the skin reaction induced by the histamine control. The test sites were placed 5 to 8 cm apart to avoid false-positive results. Histamine dihydrochloride was used as a positive control at concentrations of 0.1 % in skin prick tests or 0.01% in intradermal tests, respectively. Physiological 0.9% salime was used as a negative control. The reactions were recorded after 15 minutes by measuring the maximal longitudal and transversal diameter of the wheal and evaluated as described (66).

The use of recombinant A. fumigatus allergens for in vitro diagnostics.

The binding of recombinant A. fumigatus allergens to antibodies may be used in immunoassays for measuring allergen/antigen specific antibodies of various classes (IgA, IgG, IgD, IgE and IgM), including specific subclasses thereof, for instance in connection with diagnoses of allergy and ABPA. Among IgG subclasses may be mentioned IgG1, IgG2, IgG3 and IgG4. The methodology for the assays is the same as that used in the prior art for conventional antigens/allergens. Suitable immunoassay protocols thus contemplate formation of a ternary immune complex:

[allergen] - [anti-allergen antibody] - [anti-antibody]

where allergen and anti-antibody are added reagents and anti-allergen antibody derives from the sample to be assayed. The complex is formed in an insoluble or insolubilizable form. Insoluble forms are accomplished by having either the allergen or the anti-antibody bound to a solid phase before, after or during formation of the complex. Well known solid phases in the field are walls of tubes and wells, particulate and monolithic more or less porous materials used as adsorbents in chromatography and heterogeneous imunoassays etc. In order to measure the amount of complex, either the allergen or the anti-antibody is labelled with an analytically detectable group, with the provision that the reagent linked to a solid phase or causing post-insolubilization is not labelled. Well known detectable groups are enzymes (ELISA), fluorophors, chromophors, chemiluminescent groups, radioactive isotopes, metal atoms, biotin, haptens etc. In order to measure class/subclass specific antigen/allergen specific antibodies the anti-antibody has to be class/subclass specific.

Normally this type of immunoassay is run with sequential incubation, i.e.

step 1: sample with allergen followed by

step 2: incubation of the complex formed in step 1, i.e. [allergen] - [anti-allergen antibody] with anti-antibody

or vice versa. In case the reagent used in step 1 is bound to a solid phase, separation and washing after each step should be carried out in order to remove unspecific interference.

For ABPA diagnosis, IgE and certain IgG subclasses are the most relevant Igs to measure. It is believed that the recombinant allergens to be used should be derived from A fumigatus proteins not being exposed on the cell surface or secreted. This may indicate that the most relevant A. fumigatus allergens relevant for ABPA may be cell-bound, for instance as intracellular peptides/proteins.

Relevant antibodies can be found in blood (including plasma and serum), saliva, cerebrospinal fluid (CSF), bronchioalveolar fluid, tear drops (lacrymal fluid) etc.

The in vitro test protocols used and results.

The binding of IgE antibodies (and other isotypes) to recombinant allergens was assessed by an ELISA (39) using the same method for all allergens. Briefly, polystyrene microtiter plates were coated for 2 h at 37°C with allergen protein (10 µg/ml in PBS, pH 8.0). The free sites were blocked with PBS, pH 7.4 containing 5 % (w/v) non-fat dry milk powder (1 h, 37°C). After washing, the plates were incubated with serially twofold-diluted sera in blocking buffer containing 5% Tween 20 (2 h, 37° C). After washing, a second antibody of commercial source (66) or TN-142, a mouse monoclonal anti-human IgE antibody raised against the Cε2 domain (kindly supplied by Dr C.H. Heusser, Ciba-Geigy Ltd., Basel, Switzerland) were used to quantify the isotype-specific Ig-content of the sera. Isotype-specific Ig-binding to the allergens was detected with alkaline-phosphatase-conjugated goat anti-mouse IgG (66, 69). In absence of calibrated standards, a serum pool from two patients suffering from ABPA was used as an in house reference. Serum dilutions versus optical density were plotted in a log-log diagram and the linear titrable region used to convert the optical density values to arbitrary ELISA units (EU). Absorbence values from the reference serum pool were arbitrarily set as 100 EU/ml for all isotypes analysed (66,68).

The antigen-specific ELISA allows reliable detection of serum antibodies. For the IgE-determinations using rAsp f2, the results have been validated using Pharmacia CAP System (Pharmacia & Upjohn, Diagnostics, Uppsala, Sweden) with the recombinant proteins as immobilized allergen.

For a large scale evaluation of the in vitro diagnostic value of recombinant A. fumigatus allergens, 54 sera from patients suffering from ABPA and from 35 allergic asthmatics with A. fumigatus sensitization but without ABPA as deduced from the clinical parameters were selected. All patients had asthma and met the guidelines for the diagnosis and management of asthma (70). As negative control, sera from 10 allergic asthmatics without A. fumigatus sensitization and from 10 healthy individuals without history of atopy were used. In contrast to sera from sensitized individuals, the serum samples of the 20 control individuals showed IgE values below the background for all recombinant allergens, demonstrating that the IgE detection system is related to specific sensitization to A. fumigatus. The results of the IgE determinations obtained with sera of A. fumigatus allergic asthmatics with or without ABPA for the relevant recombinant allergens so far discovered (rAsp f2 and rAsp f4) will be discussed below.

The serological investigations rAsp f2 and rAsp f4 show a completely different picture compared to that obtained with other recombinant A. fumigatus allergens. Specific IgE against rAsp f2 and rAsp f4 was not detectable in the 35 sera from allergic asthmatics sensitized to the fungus. In contrast, the 54 sera from ABPA-patients recognized rAsp f2 and rAsp f4 at a frequency of 54% and 78%, respectively, (table 3), whereas 49 sera recognized at least one of the allergens. Therefore, serologic diagnosis of ABPA with the two allergens has a specificity of 100 % and a sensitivity > 90% (table 4). The MnSOD (rAsp f2), a protein with a known biochemical function, represents a strictly intracellular enzyme. The biobiological function of Asp f4 remains unknown; however, preliminary experiments to locate the protein using monoclonal antibodies raised against Asp f4

indicate that the protein is not secreted by the fungus. Therefore both proteins are unlikely to be present in free form as aeroallergens, which may explain the lack of specific IgE against these allergens in allergic asthmatics sensitized to A. fumigatus. In contrast, patients suffering from ABPA have or have had the fungus growing in the lung (8,12) and as a result of disintegration of fungal cells by host defence mechanisms, become exposed also to non-secreted proteins (3). One of the host defence mechanisms against fungal infections consists of the damage of hyphae and phagocytosis mediated by polymorphonuclear cells (2,3,4). Development of a cell-mediated immune response to a fungus is thought to require antigen-presenting cells to process and present fungal antigens to T-lymphocytes (1). Therefore patients suffering from ABPA are able to mount an immune response also to intracellular proteins of A. fumigatus never seen by the immune system of A. fumigatus-allergic individuals, which are exposed only to secreted allergens and conidiae. The in vivo relevance of rAsp f2 and rAsp f4 has been assessed in skin tests involving representative numbers of patients with ABPA, A. fumigatus allergy and healthy controls (see below).

Table 3. Sensitization of asthmatic patients with ABPA or A. fumigatus allergy to recombinant allergens rAsp f2 and rAsp f4.

Subject groups	rAsp f2	rAsp f4
ABPA (n=54)	54±160a	47±66
sensitized	30(56%)b	42(78%)
Allergic (n=35)	1±1	2±3
sensitized	0(0%)	0(0%)
ABPA+allergics (n=89)	33±89	29±56
sensitizedb	30(56%)	42(78%)
Healthy controls (n=20)	<5	<5
sensitized	0(0%)	0(0%)

a. Mean value of IgE-binding to the allergen ± SD (ELISA Units/ml).

b. Number and % of samples showing IgE s sensitized to the allergen above cut-off level (5 and 7 EU/ml for rAsp f4 and rAsp f4, respectively).

Table 4. Discrimination between ABPA and sensitisation to A. fumigatus by rAsp f2 and rAsp f4 IgE-specific serology.

	Number (%) individuals sensitized to allergen			
Subjects	rAsp f2	rAsp f4	rAsp f2/rAsp f4	rAsp f2+rAsp f4
ABPA (n=54)	30(56%)	42(78%)	49(91%)	25(46%)
A. fumigatus allergics (n=35)	0(0%)	0(0%)	0(0%)	0(0%)
Controls (n=20)	0(0%)	0(0%)	0(0%)	0(0%)
Specificity and sensitivity for recognition of sera from ABPA-patients				
Specificity	100%	100%	100%	100%
Sensitivity	56%	78%	91%	47%

Diagnostic value of recombinant A. fumigatus allergens for in vivo tests.

Regarding a potential discrimination between ABPA and allergic sensitization, the most significant findings of the serologic investigations, involving subjects with asthma and concomitant sensitization to A. fumigatus were elevated levels of specific serum IgE to rAsp f2 and rAsp f4 in patients suffering from ABPA. As indicated in table 3, rAsp f2- and rAsp f4-specific IgE, as measured by ELISA, reached values of 54±160 ELISA Units/ml and 47 ±66 ELISA Units/ml in sera of asthmatic patients with ABPA. In contrast, specific IgE antibodies to these two allergens were virtually absent in sera of asthmatic patients sensitized to A. fumigatus without evidence for ABPA, as well as in sera of control individuals (table 3). Based on these results, rAsp f2 and rAsp f4 could serve as reagents for the development of an

ABPA-specific assay based on circulating allergen-specific IgE antibodies. It was therefore of interest to assess the allergenicity of these proteins in vivo. To demonstrate the ability of rAsp f2 and rAsp f4 to elicit mediator release in vivo, an intradermal skin provocation study was carried out involving 12 asthmatic patients with ABPA, 12 allergic asthmatics sensitized to A. fumigatus without ABPA and 5 healthy controls. Selection of patients and diagnosis of sensitization to A. fumigatus were based on clinical history, RAST and skin reactivity to A. fumigatus extracts as described (59,66). All patients had asthma and met the guidelines for the diagnosis and management of asthma (70). At the time of the study all subjects had stable bronchial asthma, no evidence for chest infections and received no anti-histamine medication. The five healthy control individuals had no history of allergy or asthma and had normal serum levels of total IgE. The diagnosis of ABPA was based on a minimum of six of the eight criteria proposed by Rosenberg et al (23) and Patterson et al (24). Four ABPA patients (table 5) and one patient with allergic asthma (table 5) were treated with low doses of oral corticosteroids (5-10 mg/day). An ethical approval for skin testing human subjects with recombinant allergens was obtained from the responsible committee before starting the study. A full explanation of the procedure was given to all individuals before testing and subsequently a written consent was obtained. The main characteristics of the subjects participating in the study including age, sex, eosinophil count, total serum IgE, specific serum IgE to rAsp f2 and rAsp f4 and RAST to A. fumigatus are reported in table 5. All subjects showed a positive skin test response to intradermal histamine challenges (0.01%) and were non-reactive to 0.9% saline. The results (table 5) suggest a high specificity of rAsp f2 and rAsp f4 reactivity for patients suffering from ABPA. In fact, only this group of patients showed relevant amounts of specific IgE against rAsp f2 and rAsp f4 (Table 3 and 4). As expected, only individuals showing detectable amounts of allergen-specific IgE in serum reacted to skin challenges with rAsp f2 and rAsp f4. These

results clearly show that a highly specific diagnosis of ABPA based on recombinant allergens is feasible. However, although rAsp f2- and rAsp f4- based serology and skin tests show a high specificity for ABPA in the absence of atopic dermatitis, the sensitivity of the diagnosis reaches only about 90% (table 4). Taking into account the the relatively low specificity of the diagnostic criteria for ABPA available to date, serological and skin tests with rAsp f2 and rAsp f4 represent a considerable improvement of the diagnosis of the disease. Moreover, the characteristics of both allergens, taken together with the observation that ABPA patients and A. fumigatus sensitized allergic asthmatics recognize different allergen in Western blot analysis, provide a rational for a further improvement of the diagnosis of ABPA. In a study reported by Borga (6), serum IgE-reactivity to A. fumigatus allergens in two groups of patients were compared, A. fumigatus sensitized allergics and ABPA patients. Sera of individuals suffering from A. fumigatus allergy recognized at least thirty-five different IgE-binding components of the fungus, ranging between 14 and 118 kDa in size, of which four components (34, 39, 43 and 83 kDa) were uniquely detected by these sera. With sera from the ABPA group, thirty-nine different IgE-binding components ranging from 14 to 150 kDa were detected, of which eight components with molecular weights of 15, 19.5, 54, 56, 96, 110, 126 and 150 kDa were not recognized by IgE from the allergics. Therefore, from the total of 43 IgE-binding components detected, antibodies to 31 were found in both of the patient groups, 8 were specific for ABPA and 4 specific for non-ABPA-related sensitization to A. fumigatus.

The availability of the recombinant allergens described will allow identification of A. fumigatus-allergic who lack sensitization to these cloned allergens. Sera from such subjects can subsequently be used to screen the A. fumigatus phage surface display library in order to isolate phage clones displaying additional allergens. The powerful screening procedure based on biopanning (45,47,51,52), together with a rational for the selection of the sera used to screen the phage library, will

allow isolation of the additional allergens in a reasonable time. Production, characterization and evaluation of these allergens are likely to contribute to the further development specific diagnosic tools for both ABPA and A. fumigatus-related sensitization.

In order to use rAsp f2 as a specific allergen for the diagnosis of ABPA, atopic dermatitis has to be excluded. A high percentage of patients suffering from atopic dermatitis with a moderate RAST class to A. fumigatus shows high titres of rAsp f2-specific IgE in serum. Moreover, intradermal skin challenges with rAsp f2 in three patients suffering from atopic dermatitis clearly demonstrated that the allergen is able to provoke a strong in vivo mediator release in these patients. Notably the serologic investigation of 15 sera of patients suffering from atopic dermatitis does not show any specific IgE to the other recombinant A. fumigatus allergens available (76). The reason for the monovalent sensitization to Asp f2 in patients with atopic dermatitis is unknown. However, it is tempting to speculate that the specific IgE response against rAsp f2 could be due to production of IgE antibodies recognizing human superoxide dismutase in these individuals, which would result in a cross-reaction to the highly homologous fungal MnSOD (43). The availability of both the human and fungal recombinant MnSOD will allow a study of the role of these proteins in the pathophysiology of atopic dermatitis in more detail.

Table 5. Principal characteristics of the subjects studied and skin reactivity to rAsp f2 and rAsp f4.

Subject	age y	sex	Eos/ml x106	Total IgE kU/l	RAST	Specific IgE toa rAsp f2	Specific IgE toa rAsp f4	Skin test tob rAsp f2	Skin test tob rAsp f4
ABPA									
1	62	f	0.71	475	3	22	0	++	-
2	46	m	0.37	1508	4	177	48	++++	+++
3	28	m	0.45	4576	5	346	18	+++	+
4	59	f	0.35	10957	5	1	537	-	++
5	56	m	0.91	637	5	0	11	-	++

	Huvudfaxen Kassan									
	6	52	m	0.19	2476	5	45	46	++++	++
	7	55	f	0.24	1779	5	86	33	++++	++
	8	32	m	0.57	1472	4	0	82	-	++++
	9	53	m	0.23	ndc	nd	1	1	-	-
5	10	62	m	0.15	nd	nd	0	18	-	+
	11	60	f	0.08	629	4	0	47	-	+++
	12	43	m	0.53			29	8	+++	++
	A. fumigatus allergy									
	1	32	m	0.08	4913	1	4	1	-	-
10	2	60	m	0.20	76	3	0	0	-	-
	3	30	f	0.52	67	3	1	2	-	-
	4	43	f	0.05	3328	5	0	0	-	-
	5	49	f	0.75	354	3	2	0	-	-
	6	34	m	0.19	354	2	0	0	-	-
15	7	46	f	0.68	494	3	0	1	-	-
	8	59	m	0.53	116	nd	1	2	-	-
	9	58	m	0.84	>2000	3	1	1	-	-
	10	57	m	0.05	nd	nd	0	1	-	-
	11	35	f	0.41	>2000	4	0	0	-	-
20	12	44	f	0.26	1759	2	1	2	-	-
	Healthy controls									
	1	44	m	0.18	148	0	0	0	-	-
	2	33	m	0.40	25	0	1	0	-	-
	3	30	f	0.16	39	0	0	0	-	-
25	4	34	m	0.43	18	0	0	0	-	-
	5	41	m	0.26	61	0	0	1	-	-

a) Relative Elisa Units/ml

b) Positive reaction to intradermal skin challenges with rAsp f2 and rAsp f4 at concentrations of 1 µg (+), 100 ng (++), 10 ng (+++), or 1 ng (++++).

30 WHAT IS POSITIVE? DEFINITION?

c) nd 0 not determined

Serologic discrimination between sensitization to A. fumigatus and ABPA in patients with cystic fibrosis.

35 This study involved 37 patients with cystic fibrosis with routine assessment for cystic fibrosis and allergy, including

skin prick testing (67,71). 15 were diagnosed as having ABPA according to the clinical and immunological criteria proposed by Laufer (16) and Nelson (25). 12 patients belonged to the group with documented sensitization to A. fumigatus according to RAST and routine skin prick test to A. fumigatus extracts and 10 were assigned to the CF control group based on the lack of sensitization. to A. fumigatus (67). Patient characteristics including age, sex, RAST to A. fumigatus and total serum IgE are reported in table 6. Allergen specific IgE levels were determined for the allergens rAsp f1, rAsp f2, rAsp f3 and rAsp f4 in serum of each individual. rAsp f1 (43) and rAsp f3 (42) correspond to major allergens of A. fumigatus with a prevalence of sensitization of 59% and 76% among asthmatic patients with positive skin test to A. fumigatus extracts, whereas rAsp f2 (43) and rAsp f4 are recognized only by sera of patients with ABPA. All four proteins have been demonstrated to be relevant allergens in vivo by skin challenges of asthmatic patients sensitized to A. fumigatus. The results of the serological investigation (table 9) show that the majority of the cystic fibrosis individuals sensitized to A. fumigatus are carry IgE to rAsp f1 and rAsp f3 (85% and 100%, respectively) and 85% to both allergens. Taken together rAsp f1 and rAsp f3 are sufficient to diagnose sensitzation to A. fumigatus in all of the investigated sera from cystic fibrosis patients. According to the current definition of allergens (41) rAsp f1 and rAsp f3 correspond to major allergens also for the group of cystic fibrosis. In the three subgroups of individuals analyzed, cystic fibrosis patients with A. fumigatus sensitization, with or without ABPA, and cystic fibrosis individuals without A. fumigatus sensitization, relevant levels of serum IgE against rAsp f2 and rAsp f4 were found only in sera of individuals with a clinical diagnosis of ABPA. In this group rAsp f2-specific IgE levels exceeding the cut off value (> 5 U/ml) were detected in 10 of 15 patient sera, while relevant levels of rAsp f4-specific IgE (cut off value > 7 EU/ml) were detected in 13 of the 15 patient sera. If we consider elevated levels of either rAsp f2- or rAsp f4- specific IgE sufficient to

indicate ABPA, all patients were covered by the serological diagnosis, whereas 8 patients of 15 had elevated levels of IgE to both rAsp f2 and rAsp f4. Therefore allergen-specific serology with rAsp f2 and rAsp f4 could give a substantial contribution to a differential serological diagnosis of ABPA in patients suffering from cystic fibrosis and A. fumigatus sensitization.

Table 6. Serologic investigations of patients with cystic fibrosis with or without sensitization to A. fumigatus extracts.

Subject	age	sex	Total IgE	RAST	Specific IgE to rAspa			
	y		kU/l		f1	f2	f3	f4
1	11	f	2398	4	1224	1	265	76
2	12	f	5226	4	233	5	867	35
3	7	m	3804	4	747	733	4027	405
4	17	m	5423	4	173	100	658	53
5	22	f	1532	4	65	5	502	44
6	15	f	1017	4	45	30	178	13
7	16	m	ndb	4	1439	381	749	422
8	28	m	2126	4	122	23	340	17
9	12	m	866	nd	1037	60	3511	524
10	16	m	945	4	402	5	1057	27
11	28	m	576	4	208	346	287	18
12	19	m	928	3	48	97	183	4
13	16	m	nd	3	195	55	145	29
14	14	m	nd	nd	81	3	128	43
15	27	f	491	4	115	118	1529	3
A. fumigatus allergy								
1	12	m	435	4	63	5	453	2
2	30	f	554	4	45	5	21	2
3	24	f	323	4	1	1	1176	3
5	24	m	166	3	5	0	32	3
6	14	f	146	3	7	3	117	1
7	21	m	304	4	145	1	121	4
8	33	m	372	3	91	0	437	3
9	16	f	103	3	3	0	131	3
10	29	f	270	3	21	0	88	2

Huvudfaxen Kassan									
11	28	m	47	2	87	2	124	4
12	31	m	409	3	167	2	128	0
Cystic fibrosis controls									
1	20	f	56	0	2	2	9	5
2	30	m	115	0	3	4	7	2
3	30	m	nd	0	5	0	3	2
4	26	f	nd	0	1	0	1	0
5	31	m	40	0	4	0	3	2
6	32	m	59	0	6	2	4	3
7	30	m	22	0	5	1	3	2
8	36	f	nd	0	4	1	3	0
9	24	f	nd	0	1	0	4	3
10	33	m	27	0	2	0	1	0

a) Relative ELISA Units/ml

b) nd = not determined

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Sequence listings

SEQ ID NO 1:

rAsp f2 DNA sequence Length: 624

CAATACACGC TCCCACCCCT CCCCTACCCC TACGATGCCC TCCAACCCTA 50
CATCTCCCAA CAGATCATGG AGCTGCACCA CAAAAAGCAC CATCAAACCT 100
ACGTCAATGG CCTGAATGCC GCACTCGAGG CGCAGAAGAA AGCGGCGGAA 150
GCCAACGACG TCCCCAAGCT CGTCTCCGTG CAGCAAGCGA TCAAATTCAA 200
CGGCGGGGGG CACATCAACC ATTCCCTCTT CTGGAAGAAT CTGGCCCCGG 250
AGAAATCCGG GGGTGGCAAG ATCGATCAGG CACCGGTCCT CAAAGCAGCC 300
ATCGAGCAGC GTTGGGGATC CTTCGATAAG TTCAAGGATG CTTTCAACAC 350
GACCGTGCTG GGCATTCAGG GCAGCGGATG GGGTTGGTTA GTGACCGACG 400
GTCCCAAGGG AAAGCTAGAC ATTACCACAA CCCACGACCA GGATCCGGTG 450
ACCGGGGCGG CCCCCGTCTT TGGGGTGGAT ATGTGGGAGC ATGCTTACTA 500
CCTTCAGTAC TTGAACGACA AAGCCTCGTA TGCCAAGGGC ATCTGGAACG 550
TGATCAACTG GGCTGAAGCG GAGAATCGGT ACATAGCGGG TGACAAGGGT 600
GGACACCCAT TCATGAAGCT GTGA 624

rAsp f2 Amino acid sequence Length: 207

QYTLPPLPYP YDALQPYISQ QIMELHHKKH HQTYVNGLNA ALEAQKKAAE 50
ANDVPKLVSV QQAIKFNGGG HINHSLFWKN LAPEKSGGGK IDQAPVLKAA 100
IEQRWGSFDK FKDAFNTTLL GIQGSGWGWL VTDGPKGKLD ITTTHDQDPV 150
TGAAPVFGVD MWEHAYYLQY LNDKASYAKG IWNVINWAEA ENRYIAGDKG 200
GHPFMKL 207

SEQ ID NO 2:

rAsp f4 DNA sequence Length. 861

GGCGAGGTCG GCGACACTGT CTACGCTACT ATAAACGGTG TCCTCGTCTC 50
GTGGATCAAC GAGTGGTCCG GCGAGGCTAA GACCTCCGAC GCTCCCGTCT 100
CTCAGGCTAC TCCCGTCAGC AACGCTGTGG CTGCCGCCCC CGCCGCTTCT 150
ACTCCGGAGC CCAGCTCTTC CCACTCCGAC AGTTCTTCAT CCTCCGGCGT 200
CTCCGCCGAC TGGACCAACA CCCCTGCCGA AGGCGAGTAC TGCACTGACG 250

GCTTCGGTGG CAGGACCGAA CCCAGCGGCT CCGGTATCTT CTACAAGGGC 300
AACGTTGGTA AACCCTGGGG CAGCAACATC ATCGAGGTCT CCCCCGAGAA 350
CGCCAAGAAG TACAAGCACG TCGCTCAGTT TGTTGGCAGC GACACTGACC 400
CCTGGACCGT TGTCTTCTGG AACAAGATCG GCCCCGATGG TGGCCTTACT 450
GGCTGGTACG GTAACTCCGC TCTGACCCTC CACCTCGAGG CCGGTGAGAC 500
CAAGTACGTG GCATTCGACG AGAACTCCCA GGGTGCCTGG GGCGCCGCAA 550
AGGGCGACGA GCTGCCCAAG GACCAGTTTG GTGGGTACTC TTGCACCTGG 600
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CGTGTCCGCC ATTCAGGCCG AGAATGCCCA CCATGAGGTC CAGGGTATGA 700
AGATCTGCAA TCACGCCGGC GAGCTCTGCT CCATCATCTC CCACGGTCTT 750
TCCAAGGTCA TTGACGCCTA CACTGCTGAT CTGGCCGGTG TCGATGGCAT 800
TGGTGGCAAG GTCGTCCCTG GCCCTACCCG TCTGGTCGTC AACCTCGACT 850
ACAAGGAGTA G 861

rAsp f4 Amino acid sequence Length: 286

GEVGDTVYAT INGVLVSWIN EWSGEAKTSD AIVSQATPVS NAVAAAAAAS 50
TPEPSSSHSD SSSSSGVSAD WTNTPAEGEY CTDGPGGRTE PSGSGIFYKG 100
NVGKPWGSNI IEVSPENAKK YKHVAQFVGS DTDPWTVVFW NKIGPDGGLT 150
GWYGNSALTL HLEAGETKYV AFDENSQGAW GAAKGDELPK DQFDGYSCTW 200
GEFDPDSKIN HGWSGWDVSA IQAENAHHEV QGMKICNHAG ELCSIISHGL 250
SKVIDAYTAD LAGVDGIGGK VVPGPTRLVV NLDYKE 286

C L A I M S

1. A method for the diagnosis of ABPA in a human individual, characterized by determining if the individual carries antibodies reactive with one or more ABPA-related recombinant allergens.

2. The method according to claim 1, characterized in that the allergen is derived from A. fumigatus.

3. The method according to claim 2, characterized in that the allergen correspond to a non-secreted protein from A. fumigatus.

4. The method according to anyone of claims 1-3, characterized in that said one or more allergens are selected among rAsp f2 and rAsp f4 and ABPA-related fragments thereof .

5. The method according to anyone of claims 1-4, characterized in that an in vitro immunoassay is carried out on a fluid sample from the individual for the determination of the level of antibodies directed towards said recombinant allergens, in particular antibodies of the IgE class or IgG class or subclasses thereof.

6. The method according to anyone of claims 1-5, characterized in that antibodies of the IgE class are determined.

7. The method according to anyone of claims 1-4, characterized in that an in vivo test is carried out in the individual.

8. The method according to claim 7, characterized in that the test is a skin test involving placing said one or more ABPA-related allergens in the skin of the patient.

A B S T R A C T O F D I S C L O S U R E

A method for the diagnosis of ABPA in a human individual, characterized by determining if the individual carries antibodies reactive with one or more ABPA-related recombinant allergens.